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LIGHT-DEPENDENT RUBIDIUM TRANSPORT IN INTACT *HALOBACTERIUM HALOBIUM* CELLS

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SUMMARY

The uptake of rubidium in intact *Halobacterium halobium* cells was followed, and found to be light-dependent. The exchange process is slow, the steady-state rate of $^{86}Rb^+/Rb^+$ exchange being given by $k = 6.3 \cdot 10^{-4}$ min^{-1}. Starved cells exhibited a faster rate than unstarved cells. The influx of $^{86}Rb^+$ was almost completely blocked in the presence of proton conductors (CCCP, FCCP, and SF 6847), and was sensitive to the presence of the permeant cation $TPMP^+$. Valinomycin very slightly increased the rate of uptake, while $1 \cdot 10^{-6}$ M nigericin showed significant inhibition. On the other hand, release of $^{86}Rb^+$ was not light-dependent, although still affected by uncouplers, $TPMP^+$, and nigericin. These experimental observations may be explained in terms of a passive flux driven by an electrical potential difference, and influenced by positive isotope interaction within the membrane. In carefully matched influx-efflux studies, the extent of the positive isotope interaction was measured. Using the formal treatment of Kedem and Essig, the ratio (exchange resistance)/(resistance to net flow) for $^{86}Rb^+$ was found to be 1.7.

INTRODUCTION

The halophilic bacterium *Halobacterium halobium* utilizes solar energy by synthesizing a purple protein, bacteriorhodopsin, which absorbs light and thereby establishes a proton electrochemical potential difference across the cell membrane [1–3]. In accordance with Mitchell's chemiosmotic hypothesis [4, 5] this gradient drives ATP synthesis [6–9].

A unique property of the halophilic bacteria is their ability to concentrate up to 4–5 M potassium internally against a large concentration gradient [10–12]. Studies on an unidentified species of halobacteria isolated from the Dead Sea led Ginzburg et al. to conclude that most of the internal potassium is tightly and specifically bound to the

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid); SF6847, 3,5-di tert butyl-4-hydroxybenzyliden malononitrile; $TPMP^+$, triphenyl methyl phosphonium.

cell matrix while the membrane is highly permeable to small ions [10, 11, 13, 14]. On the other hand, since no bound potassium was detected in lysed *Halobacterium cutirubrum* cells, Lanyi and Silverman suggested that most of the internal potassium is chemically active and held inside by an impermeable cell membrane [12].

In this paper, a study of the transport of Rb^+ and K^+ through the cell membrane was carried out. Rb^+ is a common analogue for K^+ in biological systems, and because of its technical convenience $^{86}Rb^+$ was employed for the transport studies using cells grown in a Rb^+ medium. Although we report only data obtained with $^{86}Rb^+$, preliminary studies with $^{42}K^+$ show similar behaviour.

METHODS AND MATERIALS

Cell cultures. Strain R_1 of *H. halobium* [15] was grown as described previously [6]. Cultures were raised aerobically on a synthetic medium [16]. After they reached the stationary phase, they were diluted 40 times with medium in which KCl was substituted by RbCl at the same molarity. The diluted cultures were raised for an additional 4 days in semi-anaerobic conditions exposed to strong illumination.

Uptake measurements. Four-day cultures, raised on RbCl, were centrifuged at $6000 \times g$ for 10 min and twice resuspended in basal salt solution at pH 6.7. The salt solution contained 4 M NaCl, 30 mM RbCl, 80 mM $MgSO_4$, and 0.1 mM $CaCl_2$. The cell suspensions (in basal salt solution) contained 5–7 mg cellular protein/ml suspension, determined according to Lowry et al. [17], and 15–30 nmol bacteriorhodopsin/ml suspension, determined by the method of Danon and Stoeckenius [6], assuming a molar extinction coefficient of 63 000 $M^{-1}\ cm^{-1}$ for bacteriorhodopsin at 570 nm [18]. Cell suspensions were starved by stirring them overnight in the dark in the presence of 0.5 μg/ml DNAase. The enzyme was added to decrease the high viscosity due to DNA released from lysed cells. The starved cells were spun down at $6000 \times g$ once more and resuspended in the same volume of basal salt solution containing 50 mM PIPES buffer (pH 6.5–7.5), 15 μCi ^{86}RbCl/ml, and 100 μCi ^3H_2O/ml.

The suspension was divided among several stirred glass vessels, located in the front of the light source, the appropriate agents were added, and the light was switched on ($t = 0$). Two 250 W reflector lamps served as the light source, and 3-cm thick water filters were used to avoid heating the vessels. The light intensity in the vessels was $5 \cdot 10^5\ erg \cdot cm^{-2} \cdot s^{-1}$. Sampling was done by the microcentrifugation technique. At different times aliquots of 200 μl were taken in triplicate from each vessel into 400-μl microfuge tubes, and spun down for 5 min in a Beckman 152 microfuge. A pellet fraction, obtained by cutting through the pellet, and a 10 μl sample from the supernatant of each tube, were transferred to test tubes and 0.5 ml 1 M NaOH was added. After dissolution aliquots of 10 μl were taken into scintillation vials and 10 ml scintillation fluid, prepared according to Bray [19], were added. ^{86}Rb and ^{3}H were counted simultaneously in a Tri carb liquid scintillation spectrometer (Packard model 3320). Steady state influx (needed to measure the rate constant of $^{86}Rb^+$ exchange) was followed in unstarved suspensions, the culture having been resuspended in its growth medium once without being incubated overnight. The activity of $^{86}Rb^+$ in the pellet was corrected for the tracer present in the trapped volume between cells (non-osmotic space), and expressed as mM $^{86}Rb^+$ in the cells, normalized to a specific activity outside of 1 at $t = 0$. The internal water space and the water content of the

pellet were measured with ^3H_2O and [^{14}C]sucrose as described in ref. 20.

Estimation of $\Delta\psi$. The transmembrane electrical potential ($\Delta\psi$) was estimated in intact cells as described by Bakker et al. [20], using [^{3}H]TPMPBr.

Efflux measurements. Concentrated cell suspensions were prepared as described above, and stirred overnight with 0.5 mg DNAase/ml suspension and 60 μCi ^{86}RbCl/ml suspension exposed to strong illumination. The loaded cells were spun down at $6000 \times g$ and resuspended in the same volume of unlabelled salt solution. After incubation for 30 min in the light, they were spun down once more and resuspended in basal salt solution containing 50 mM PIPES, $1 \cdot 10^{-2}$ M NaN_3 and 10 μCi/ml ^3H_2O. The suspensions were divided among several vessels, different drugs were added, and light was switched on. Supernatant sampling was done by centrifuging aliquots of 100 μl and taking triplicates of 10 μl supernatant from a single microfuge tube directly to the scintillation vials. The pellet was sampled as described earlier. Since in this set of experiments label accumulation in the supernatant was followed, interpretation of the results as mM $^{86}Rb^+$ released from the cells involved the ratio between the internal and external volumes. This number was determined by measuring cellular H_2O/mg protein (the internal water space) and mg pellet protein/ml suspension for each preparation.

Sources of chemicals and radioisotopes. CCCP, valinomycin, PIPES, and DNAase were obtained from Sigma Chemical Co; DCCD from Fluka; TPMPBr from K and K Laboratories; nigericin from Eli Lilly and Co.; SF6847 was a gift from Dr. Y. Nishizawa, Sumimoto Chemical Industry, Osaka (Japan). [^{3}H]TPMP (114 Ci/mol) was a gift from Dr. R. Kaback, Roche Institute of Molecular Biology, Nutley, N.J. (U.S.A.); [^{14}C]sucrose, ^3H_2O and ^{86}RbCl were obtained from Amersham Radiochemical Centre, U.K.

RESULTS

Uptake of $^{86}Rb^+$ by intact cells

Fig. 1a shows uptake of $^{86}Rb^+$ by *H. halobium* cells which were starved of substrates overnight in the dark. $^{86}Rb^+$ uptake is due to net Rb^+ influx and/or exchange of the tracer for the abundant ion. The accumulation of labelled material is a very slow process and more than 24 h were required to equilibrate the specific activities on both sides of the membrane. The uptake was found to be light dependent; switching off the light reduced the uptake rate drastically. Similar observations in membrane vesicles from *H. halobium* were made by Kanner and Racker [21]. Re-illumination of cells which had been in darkness restored the rate of $^{86}Rb^+$ uptake. When the overnight starvation was carried out under light, the same light-dependent process was obtained but the rates were significantly lower (Fig. 1b).

To characterize the tracer exchange kinetically, the relaxation constant and the half-time of $^{86}Rb^+/Rb^+$ exchange were measured. Tracer influx was followed in unstarved cells, as described under Methods. Since no flux was observed in cells which were grown on radioactive rubidium (and therefore have the same specific activity on both sides of the membrane) the accumulation of labelled material in the cells under these conditions reflects self-exchange only. The kinetic parameters were calculated according to Solomon [22], assuming a closed two-compartment system and a uniform distribution of Rb^+ in each compartment. The values obtained were

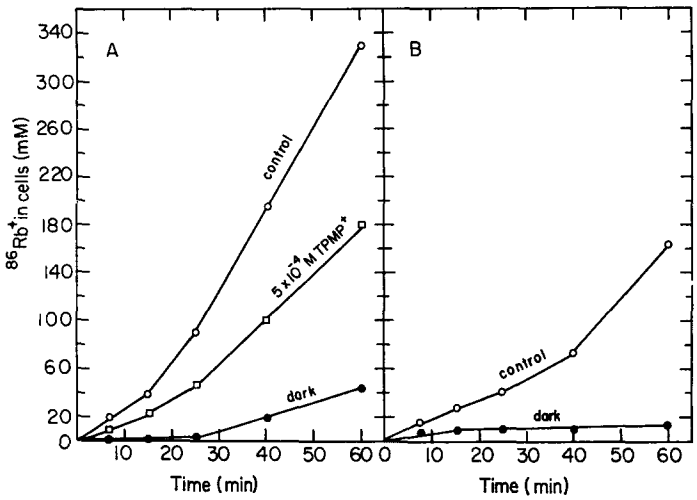

Fig. 1. $^{86}Rb^+$ uptake by intact cells. A. 9 ml of concentrated cell suspension (7 mg protein/ml) were starved as described under Methods and Materials. After the overnight preincubation they were spun down and resuspended in a mixture containing 7 ml basal salt, 1.8 ml 250 mM PIPES (pH 6.7, dissolved in basal salt), 135 μCi ^{86}RbCl and 0.9 mCi ^3H_2O. The cells were then transferred to three vessels (3 ml in each). ○—○, 150 μl basal salt were added and light was switched on; ●—●, 150 μl basal salt were added and the vessel remained in the dark; □—□, 150 μl 10^{-2} M TPMPBr (dissolved in basal salt) were added and light was switched on. B. 6 ml of the same suspension were treated as before except that the overnight preincubation was carried out in the light. The resuspension was done in the following mixture: 4.8 ml basal salt, 1.2 ml 250 mM PIPES, 95 μCi ^{86}RbCl, and 600 μCi ^3H_2O. The cells were transferred to two vessels, ○—○, in the light; ●—●, in the dark.

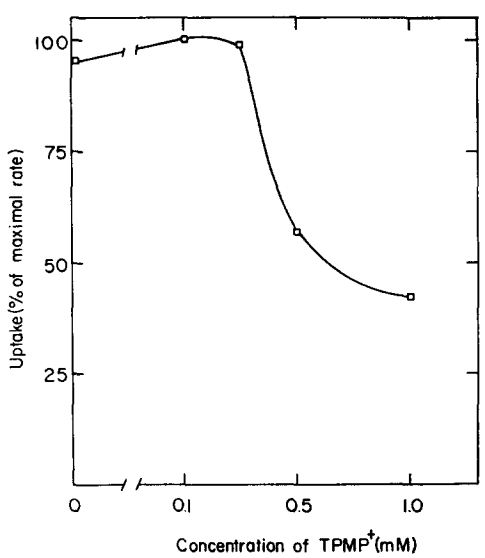

Fig. 2. The influence of $TPMP^+$. Cells were starved and resuspended in labelled basal salt solution as described before. After resuspension equal volumes of TPMPBr dissolved in basal salt (at different concentrations) were added and the influx of $^{86}Rb^+$ for different $TPMP^+$ concentrations was followed. The rates of tracer influx were determined and the results were expressed as percentage of the maximal rate.

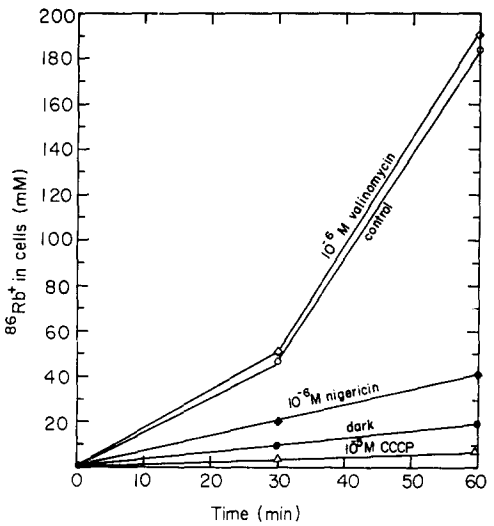

Fig. 3. Kinetics of $^{86}Rb^+$ influx. Suspensions were treated as described earlier. After resuspension with labelled rubidium, 2-ml aliquots were placed in vessels in front of the light source, and the following drugs were added: ○—○, 2 μl alcohol; ●—●, 2 μl alcohol and the vessel was covered with aluminium foil (dark vessel); △—△, 2 μl 10^{-2} M CCCP (final concentration 10^{-5} M); ◆—◆, 2 μl 10^{-3} M nigericin (final concentration 10^{-6} M); ◇—◇, 2 μl 10^{-3} M valinomycin (final concentration 10^{-6} M). All the drugs were dissolved in alcohol.

$k = 6.3 \cdot 10^{-4}$ min^{-1} and $t_{\frac{1}{2}} = 13.5$ h. These are representative numbers, since the actual values of k and $t_{\frac{1}{2}}$ varied markedly from culture to culture.

Triphenyl methyl phosphonium ($TPMP^+$) is a synthetic cation which can penetrate through lipid membranes [23]. Bakker et al. [20] showed that at concentrations higher than 10^{-4} M, $TPMP^+$ reduces the transmembrane potential difference, but does not affect or even increase the proton electrochemical potential difference (since the decrease in $\Delta\psi$ is associated with an increase in ΔpH). A concentration of $5 \cdot 10^{-4}$ M $TPMP^+$ decreased the rate of $^{86}Rb^+$ uptake by starved cells to 50 % of its initial value (Fig. 1a). Fig. 2 shows the concentration dependence of this effect. Less than 10^{-4} M $TPMP^+$ does not inhibit (and even slightly increases) $^{86}Rb^+$ influx, but when higher concentrations are introduced a steep reduction of the uptake rate takes place. More than 10^{-3} M $TPMP^+$ could not be applied since the cells failed to form tight pellets.

Proton conductors were found to have a strong effect on $^{86}Rb^+$ uptake by starved cells. CCCP at 10^{-5} M almost completely stopped any accumulation of labelled material in the cells (Fig. 3). FCCP and SF 6847 had similar effects. These uncouplers have been shown to collapse $\Delta\psi$ [20]. Potassium and rubidium carriers were also tested. Nigericin, which is known to exchange K^+ or Rb^+ for H^+, inhibited the uptake quite efficiently at 10^{-6} M, but 10^{-6} M valinomycin, which is a specific Rb^+ and K^+ conductor had (if anything) a very small accelerating effect on the labelled rubidium influx. When the normal uptake was poor, e.g. in cultures which were too 'old' or contained more than 1 % (v/v) alcohol in the suspension, 10^{-6} M valinomycin stimulated the uptake by 2–3 fold. 10^{-5} M DCCD, which is a specific ATPase inhibitor, did not affect the uptake (not shown).

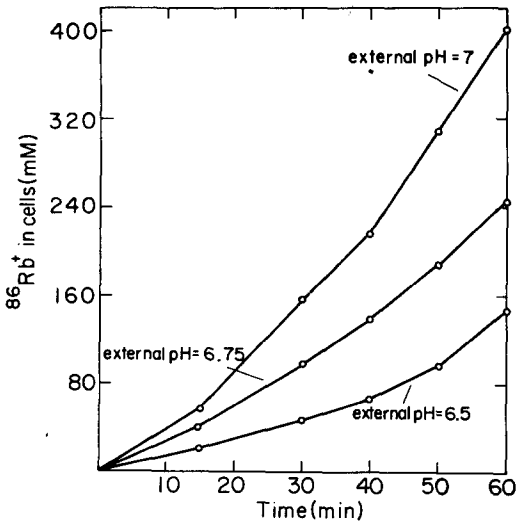

Fig. 4. pH dependence of $^{86}Rb^{+}$ influx. A bacterial suspension was starved as described earlier. A 12 ml aliquot was spun down and resuspended in basal salt to a final volume of 0.6 ml. 0.9 mCi $^{3}H_2O$ and 135 μCi $^{86}RbCl$ were added in the dark and the cells transferred to three vessels, 3.2 ml in each, which contained 0.8 ml 250 mM PIPES buffer at different pH values. Light was switched on and tracer uptake was followed as described under Methods and Materials.

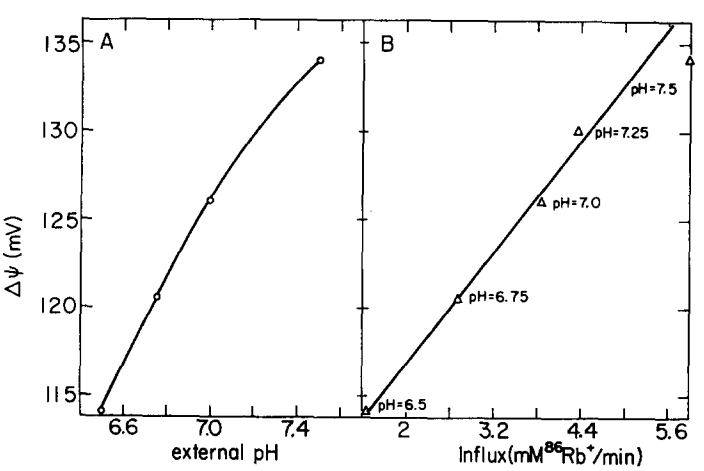

Fig. 5. $\Delta\psi$ dependence of $^{86}Rb^{+}$ uptake. $^{86}Rb^{+}$ influx was followed at different external pH values as described in the legend to Fig. 4. The transmembrane potential difference was measured for the same batch of cells, as described under Methods and Materials. A. $\Delta\psi$ plotted against the external pH; B. $\Delta\psi$ plotted against initial rates of $^{86}Rb^{+}$ influx.

An additional study was made of ^{42}K uptake by starved cells grown on KCl (Cooper, Garty and Caplan, unpublished results). The findings were similar to those obtained with $^{86}Rb^+$, except that nigericin increased the rate of ^{42}K uptake.

The rate of $^{86}Rb^+$ uptake was found to be influenced by the external pH, the tracer being accumulated faster when the pH of the suspension was increased (Fig. 4). Since it was shown that the transmembrane potential difference in *H. halobium* is also increased by increasing the external pH [20], it was of interest to see whether the change in the ion flux correlates with the change in $\Delta\psi$. In Fig. 5 the initial rate of $^{86}Rb^+$ uptake was plotted against the transmembrane potential difference for different values of the external pH. It is seen that influx is linearly dependent on $\Delta\psi$ in the range of 6.5–7.25 (more extreme pH values were not checked).

Efflux measurements

Like influx, release of $^{86}Rb^+$ by loaded cells is a slow process which is essentially linear with time during the first hour (Fig. 6) although in some experiments a seemingly faster initial release was observed.

A much faster initial release was seen if the cells were not stirred for 30 min between the resuspensions (Fig. 7), and this initial fast release was obtained even if the

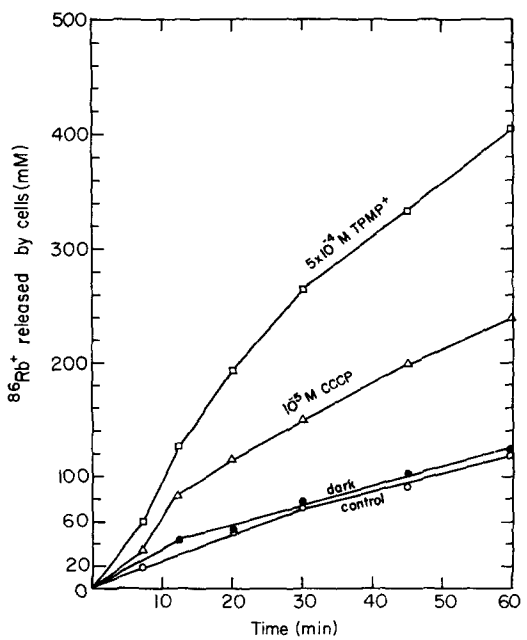

Fig. 6. Efflux of $^{86}Rb^+$. Cells were loaded as described under Methods and Materials. Loaded cells were centrifuged and resuspended in unlabelled basal salt solution. After 30 min of stirring they were spun down again and resuspended in a buffered solution of basal salt containing 10 μCi H_2O/ml suspension and 10^{-2} M NaN_3 (to block residual oxidative phosphorylation). 1-ml aliquots were placed in the experimental vessels and the following drugs were added: ○—○, 50 μl basal salt+1 μl alcohol (control vessel); ●—●, 50 μl basal salt+1 μl alcohol, and the vessel was covered with aluminium foil (dark sample); △—△, 50 μl basal salt+1 μl 10 M CCCP (final concentration 10^{-5} M); □—□, 50 μl 10^{-2} M TPMPBr dissolved in basal salt (final concentration $5 \cdot 10^{-4}$ M)+1 μl alcohol.

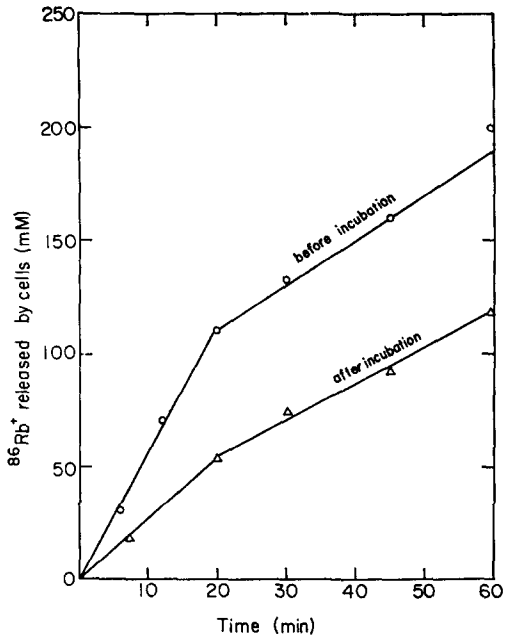

Fig. 7. Effect of incubation on $^{86}Rb^{+}$ efflux. Efflux was measured as described earlier. △—△, cells were incubated for 30 min between the two resuspensions; ○—○, cells were resuspended twice without any incubation in the unlabelled medium.

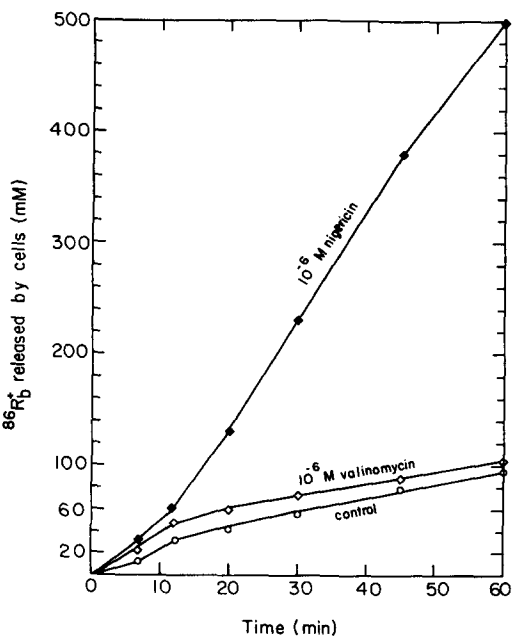

Fig. 8. Influence of ionophores on $^{86}Rb^{+}$ efflux. The same procedure was carried out as in Fig. 6. The drugs added were: ○—○, 1 μl alcohol; ◇—◇, 1 μl 10^{-3} M valinomycin; ◆—◆, 1 μl 10^{-3} M nigericin (final ionophore concentrations 10^{-6} M).

cells were resuspended in labelled basal salt less radioactive than the incubating medium, but still containing $^{86}Rb^+$ at higher specific activity than the interior. We therefore conclude that the relatively fast initial efflux of $^{86}Rb^+$ does not represent transport through the membrane, but is merely an artifact due to release of $^{86}Rb^+$ which was adsorbed on the outer side of the membrane during the overnight incubation. We find it important to emphasize this point in the light of the possibility that part of the internal K^+ or Rb^+ equilibriates rapidly with the external medium [11, 13]. Radioactive rubidium could not be eliminated completely from the external medium; lysing of cells during resuspension and squeezing of the cells by centrifugation resulted in substantial amounts of $^{86}Rb^+$ being present in the medium at $t = 0$. Therefore effluxes were measured much closer to equilibrium than uptake of $^{86}Rb^+$ and their rates are not comparable.

It was found that agents which inhibited $^{86}Rb^+$ uptake accelerated $^{86}Rb^+$ release from loaded cells (Figs. 6 and 8). Uncouplers like CCCP which prevented uptake almost completely increased release of $^{86}Rb^+$ from loaded cells. $TPMP^+$ ($5 \cdot 10^{-4}$ M) had a very marked effect, being more effective in accelerating rubidium efflux than 10^{-5} M CCCP. Unexpected are the tiny differences between the efflux in the light and in the dark, in contrast to the influx measurements. We refer to this point in the discussion. Valinomycin again had a small accelerating effect on the unidirectional flow, and nigericin stimulated efflux five fold.

A useful relation between isotope fluxes measured under different driving forces arises from Kedem and Essig's flux ratio equation [24]:

$$\frac{\text{influx}}{\text{efflux}} = \frac{J_2/\Delta\rho_2}{-J_3/\Delta\rho_3} = \exp\frac{R^\star}{R}\cdot\frac{X-\int_0^{\Delta x}\sum_{j=4}^{n} r_{0j}J_j\,dx}{RT} \tag{1}$$

Here X represents the negative electrochemical potential difference of the transported ion across the membrane of thickness Δx, J_2 and J_3 are tracer isotope flows corresponding to influx and efflux (J_1 would be the flow of the abundant species), J_j ($j \geqslant 4$) represents flows or rates of metabolic reactions to which the net flow of the test species J ($= J_1+J_2+J_3$) may be coupled (the corresponding coupling coefficient being r_{0j}), $R^\star$ and R are the exchange resistance and the resistance to net flow, and $\Delta\rho_2$ and $\Delta\rho_3$ the differences in tracer specific activities. It should be pointed out that Eqn. 1 does not require linear relationships between flows and forces [24], and near equilibrium it applies even in the case of non-continuous electrochemical potential gradients [25].

If the tracer fluxes are completely independent, $R^\star$ should be equal to R. $R^\star/R \neq 1$ indicates interaction between the influx and the efflux in the membrane ($R^\star/R > 1$ for positive interaction and $R^\star/R < 1$ for negative interaction).

If fluxes are measured in two different states A and B, Eqn. 1 can be written for each state separately, and by dividing the two expressions one obtains

$$\frac{\text{influx}_A}{\text{influx}_B}\cdot\frac{\text{efflux}_B}{\text{efflux}_A} = \exp\left\{\frac{R^\star}{R}\,\frac{(X^A-X^B)-\int_0^{\Delta x}\sum_{j=4}^{n} r_{0j}(J_j^A-J_j^B)\,dx}{RT}\right\} \tag{2}$$

Eqn. 2 provides a method of detecting the presence of isotope interaction and of

evaluating it if the test species can be assumed to be completely uncoupled to other flows. In this case one can measure tracer fluxes for different values of X and plot ln $[(\text{influx}_A/\text{influx}_B)\cdot(\text{efflux}_B/\text{efflux}_A)]$ versus $X^A - X^B$. Such a plot should yield a straight line whose slope is $(R^*/R)/(RT)$. The advantage of Eqn. 2 over Eqn. 1 is that it involves $X^A - X^B$ rather than X. It is usually easier to estimate a change in driving force than to determine its absolute value. For example, the estimation of X involves the activity coefficient of the test species inside the cell, while the difference $X^A - X^B$ may involve purely electrical parameters. A procedure for determining R^*/R from unidirectional flux measurements at two settings of X was suggested previously by Li et al. [26]. However, we preferred not to use their procedure since it involves knowing the absolute value of the electrochemical potential differences as in the case of Eqn. 1.

The above procedure was carried out for Rb^+ in intact *H. halobium* cells. Changes in X were induced by buffering the external medium at different pH values and consequently varying $\Delta\psi$ (as shown in Fig. 5). Since Rb^+ has only one tracer, simultaneous measurement of influx and efflux was impossible. Tracer fluxes were therefore followed in matched experiments, and values of $\Delta\psi$ determined for the same batch of cells at each external pH chosen. The efflux turned out to be hardly affected by the external pH, $\ln(\text{efflux}_B/\text{efflux}_A)$ being practically zero. Values of $(\text{influx}_A/\text{influx}_B)$ were calculated from the initial rates of $^{86}Rb^+$ uptake at different pH values, and since

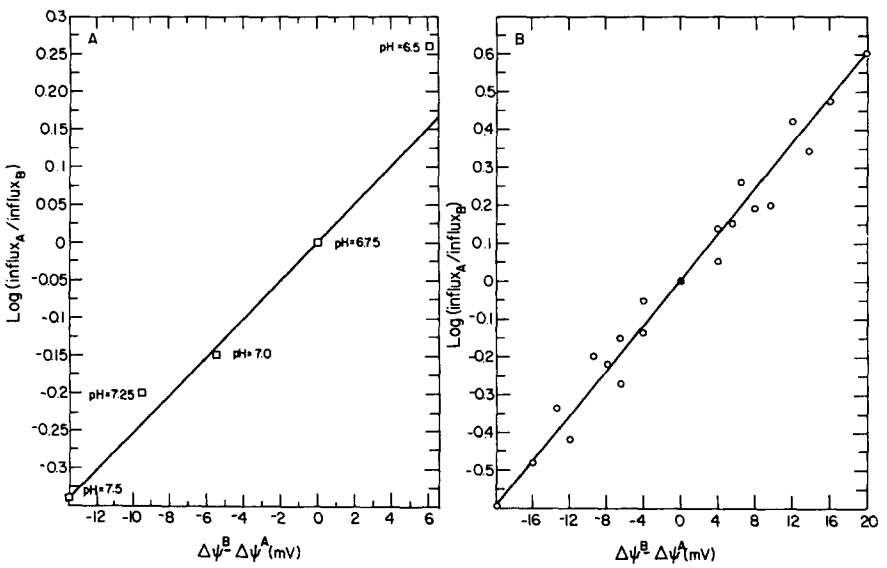

Fig. 9. Thermodynamic analysis of tracer fluxes. Two aliquots from the same batch of cells were incubated in the light, one with 60 μCi ^{86}RbCl/ml. After 12 h they were spun down and resuspended in basal salt, incubated for another 30 min in the light, and part of the unlabelled suspension was taken for $\Delta\psi$ determination, the rest were spun down again and resuspended in basal salt solution buffered at pH 6.5–7.5 with 50 mM PIPES containing 100 μCi/ml ^3H_2O. ^{86}RbCl was added to the aliquot that preincubated in the unlabelled medium, and tracer influx and efflux were followed simultaneously. A. Influx_A and $\Delta\psi^A$ were arbitrarily designated as the values measured at pH 6.75, influx_B and $\Delta\psi^B$ were successively taken to be the values measured at other pH values. B. A plot of the same data as in A, but here each pH value in turn was taken to represent state A while the remaining pH values represented successive states B.

no significant change in the chemical gradient of Rb^+ can take place during the short time after buffering of the external medium, $X^A - X^B$ was taken as $F(\Delta\psi^B - \Delta\psi^A)$. Plotting $\log(\text{influx}_A/\text{influx}_B)$ against $\Delta\psi^B - \Delta\psi^A$ gave the result shown in Fig. 9. The points on the plot can be fitted rather well to a straight line, the correlation coefficient being 0.98. The ratio R^*/R determined from the slope is 1.7 ± 0.34 S.E.

Bakker et al. [20] pointed out that the distribution of $[^3H]TPMP^+$ is not an accurate measurement of the transmembrane electrical potential since the labelled cation tends to accumulate in the membrane. Although the value of $\Delta\psi$ may contain a significant error, that does not affect the change in $\Delta\psi$ with external pH and this method should give a good estimate of the difference $\Delta\psi^B - \Delta\psi^A$. In evaluating R^*/R it was assumed that the flow of Rb^+ is uncoupled to other processes (i.e. $r_{0j} = 0$ for $j \geqslant 4$). However, this assumption may be unjustified. While the thermodynamic analysis does not eliminate the possibility of such coupling, it indicates that positive isotope interaction may account satisfactorily for the observations.

DISCUSSION

Uptake of rubidium by *H. halobium* cells was found to be a slow process. The rate constant for steady state Rb^+ exchange is of the same order of magnitude as encountered in other bacteria (e.g. $0.6–1.5 \cdot 10^{-3}$ min^{-1}, depending on the pH, for the wild type *Streptococcus faecalis* [27]). Slow uptake of ^{42}K by an unidentified species of halobacteria was reported previously by Ginzburg et al. [11]. These workers suggested that most of the inner K^+ is tightly bound, and that only a small free pool exists which is at equilibrium, through a highly permeable membrane, with the external medium. In this view the slow uptake of labelled potassium observed is due to slow exchange between the free and bound pools inside the cell. Our studies suggest that the slow uptake of both $^{86}Rb^+$ and $^{42}K^+$ is due to an impermeable membrane rather than to slow exchange inside the cell. Since the uptake does not appear to exhibit biphasic kinetics, one can assume either that it is a very slow process, or that it is a very fast one such that the internal pool equilibriates with the external medium before the first sample is taken. The second possibility seems to us unlikely; according to Fig. 1 a fast, pre-equilibrating pool would be very small and beyond the resolution of the experiment. Furthermore it is hard to see how CCCP, $TPMP^+$, and nigericin can all change the state of binding. Their action is explained much more readily by means of effects on the membrane permeability and the transmembrane potential. Although the experimental observations do not rule out the possibility of binding inside the cell, it seems that tracer uptake by intact cells is limited by the membrane permeability rather than internal exchange. Since the transmembrane electrical potential difference in *H. halobium* cells at physiological pH was estimated to be between 95 and 116 mV [20], it is not necessary to assume a large binding to account for the high internal concentration of K^+. At equilibrium the K^+ chemical activities are related to the transmembrane potential by $\ln(a_{in}/a_{out}) = -(F\Delta\psi/RT)$. Inserting $\Delta\psi = -116$ mV yields $a_{in}/a_{out} = 100$. Thus the external 30 mM K^+ should be balanced internally by 3 M chemically active K^+ under equilibrium conditions.

Both $^{86}Rb^+$ and $^{42}K^+$ transport showed a very clear dependence on energy, but seemed not to be directly coupled to ATP. The process required light and was inhibited by uncouplers, but was unaffected by 10^{-5} M DCCD. Energy-dependent

$^{86}Rb^+$ and $^{42}K^+$ autologous exchange has been observed in other bacteria. It was shown [27, 28] that uptake of $^{86}Rb^+$ and $^{42}K^+$ in intact *S. faecalis* cells requires metabolic energy and is inhibited by proton conductors. In membrane vesicles of *Escherichia coli*, *Staphylococcus aureus*, and *Micrococcus denitrificans*, Rb^+ uptake required electron donors and was inhibited by anaerobiosis or electron transfer inhibitors [29].

Two different explanations have been suggested for the close link between the K^+ or Rb^+ transport and metabolism. Harold and Altendorf suggested that in *S. faecalis* these ions are transported passively, driven by the $\Delta\psi$ generated by proton extrusion [30]. On the other hand, the requirement of an energy source for Rb^+ transport in *E. coli* was understood in terms of active transport. According to Lombardi et al. [29], K^+ is pumped inwards by an electrogenic process and the positive potential generated drives H^+ and Na^+ out. This model is clearly untenable in *H. halobium*, since it is well-established that protons are ejected actively from the cell by means of a light-driven pump, and that the electrical potential generated is negative inside [3, 6–8, 20].

We suggest that in *H. halobium*, as in *S. faecalis*, Rb^+ and K^+ equilibrate according to the value of $\Delta\psi$, which otherwise drives a net flux of these ions. When cells are preincubated in basal salt in the dark, they utilize their internal substrates. When they run out of substrates $\Delta\psi$ drops and as a result cations leak out. Switching on the light after overnight starvation in the dark builds up the transmembrane potential and net influx of cations takes place. $^{86}Rb^+$ accumulation by cells under these conditions reflects tracer uptake in the presence of net influx. On the other hand, when cells are illuminated during the preincubation $\Delta\psi$ does not drop. Substrates are still utilized since light inhibits only 30 % of respiration [31]; the decrease in the rate of oxidative phosphorylation is compensated for by photophosphorylation. Thus $^{86}Rb^+$ accumulation in cells under these conditions reflects tracer uptake in the absence of net flux and consequently its rate is smaller than in the previous case, as shown in Fig. 1. When $^{86}Rb^+$ influx is subsequently measured in the dark one follows tracer uptake against net efflux. The decrease in $\Delta\psi$ estimated by Bakker et al. [20] cannot account for the tremendous effect on influx observed on switching off the light, but positive isotope interaction appears to provide an explanation. In the same way the inhibition of $^{86}Rb^+$ uptake by $TPMP^+$ and CCCP can be understood. Both of these agents decrease $\Delta\psi$ [20] and therefore induce net Rb^+ efflux. This efflux interferes with tracer influx. The rates of ^{86}Rb uptake measured under different conditions are in quantitative agreement with the values of $\Delta\psi$ measured under the same conditions: CCCP < dark < $TPMP^+$ < control.

A quite different pattern was obtained when release of $^{86}Rb^+$ was followed. In this case the sequence of rates was $TPMP^+$ > CCCP > dark ⩾ control. The increased effectiveness of $TPMP^+$ here as compared with the influx studies may indicate exchange of Rb^+ for $TPMP^+$. The difference in the light effect between efflux and influx can be understood in terms of Eqn. 2. From this equation it is clear that the ratio $influx_A/influx_B$ is not necessarily equal to the ratio $efflux_A/efflux_B$; an asymmetry in the sensitivities of influx and efflux to light is allowed.

The isotope interaction observed gives some indication of the mechanism by which Rb^+ is transported in *H. halobium*. According to ref. 25, carrier mechanisms give rise to negative isotope interactions while channel mechanisms which allow 'friction'

among the transported species [32] give rise to positive isotope interaction. Since $R^*/R > 1$ we conclude that Rb^+ and K^+ are not transported in *H. halobium* by means of a simple carrier.

In our system nigericin decreases $^{86}Rb^+$ uptake by the cells but has the opposite effect on $^{42}K^+$ uptake. Rb^+ release was accelerated by the addition of 10^{-6} M nigericin. This behaviour may result from the difference in the affinities of nigericin for K^+ and Rb^+ [33]. In principle nigericin stimulates the self-exchange of $^{86}Rb^+$ and $^{42}K^+$ as well as the net efflux of these ions. In the case of $^{42}K^+$ the amount taken up by self-exchange is apparently greater than the amount exchanged for H^+, and the total effect is an increase in the rate of tracer accumulation. On the other hand, since the affinity of nigericin for Rb^+ is considerably smaller than its affinity for K^+, self-exchange of Rb^+ seems to be less important and net $^{86}Rb^+$ release is the predominant process.

Although the cell membrane is essentially cation impermeable, valinomycin was found to be ineffective both on uptake and release of Rb^+. This ineffectiveness is not due to the fact that Rb^+ was substituted for K^+ since this drug has nearly the same selectivity to both ions [33], and experiments with K^+ gave the same results. Valinomycin was also found to be ineffective on the extremely halophilic species isolated from the Dead Sea [13]. On the other hand, Kanner and Racker [21] reported a 3–7-fold accumulation in the rate of Rb^+ uptake by *H. halobium* vesicles induced by $3 \cdot 10^{-6}$ M valinomycin. Some indirect effects of this antibiotic have also been observed. Danon and Caplan [9] stimulated ATP synthesis in intact *H. halobium* cells by acidification and addition of valinomycin at low external K^+ levels, and MacDonald and Lanyi [34] inhibited leucine uptake in membrane vesicles by 10^{-6} M valinomycin. These indirect effects do not contradict our results, since they probably involve a net flux of very few K^+, which influences $\Delta\psi$ but is too small to affect tracer distribution. Even the direct effect on Rb^+ measured by Kanner and Racker [21] is a small one compared to what is observed in other biological and artificial systems, (see for example, ref. 35). So it appears that valinomycin has a limited effect on the membrane of halophilic bacteria. It is not clear to us why this is so, but a possible explanation is that the high salt concentration affects somehow the ionophore conformation and reduces its ability to carry K^+ and Rb^+. Another possible effect of the high salt concentration might be to change the partition coefficient of valinomycin between the aqueous and the lipid phases, and to reduce its actual concentration in the membrane (although this is probably a minor effect).

Our studies of intact *H. halobium* cells suggest that although the ion composition of this extremely halophilic microorganism differs markedly from that of other bacteria, the Rb^+ and K^+ transport mechanisms through the cell membrane are not unusual. The results do not rule out the possibility of active transport, but all the findings can be explained by means of passive transport through a comparatively impermeable membrane, driven by the electrical gradient as suggested by Harold and Altendorf for *S. faecalis* [30] and earlier by Rottenberg for K^+ transport in mitochondria [36].

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